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RESEARCH LETTER

Genome mining in *Amycolatopsis balhimycina* for ferredoxins capable of supporting cytochrome P450 enzymes involved in glycopeptide antibiotic biosynthesis

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Abstract

Ferredoxins are required to supply electrons to the cytochrome P450 enzymes involved in cross-linking reactions during the biosynthesis of the glycopeptide antibiotics balhimycin and vancomycin. However, the biosynthetic gene clusters for these antibiotics contain no ferredoxin- or ferredoxin reductase-like genes. In a search for potential ferredoxin partners for these P450s, here, we report an *in silico* analysis of the draft genome sequence of the balhimycin producer *Amycolatopsis balhimycina*, which revealed 11 putative Fe–S-containing ferredoxin genes. We show that two members (balFd-V and balFd-VII), produced as native-like *holo*-[3Fe–4S] ferredoxins in *Escherichia coli*, could supply electrons to the P450 OxyB (CYP165B) from both *A. balhimycina* and the vancomycin producer *Amycolatopsis orientalis*, and support *in vitro* turnover of peptidyl carrier protein-bound peptide substrates into monocyclic cross-linked products. These results show that ferredoxins encoded in the antibiotic-producing strain can act in a degenerate manner in supporting the catalytic functions of glycopeptide biosynthetic P450 enzymes from the same as well as heterologous gene clusters.

Introduction

Cytochrome P450 enzymes typically catalyze the hydroxylation of substrates, using molecular oxygen and reducing equivalents supplied by NAD(P)H. The class I bacterial cytochrome P450 hydroxylases require a flavin-dependent ferredoxin reductase (FdR), which is reduced by NAD(P)H, and a ferredoxin (Fd) iron–sulfur protein to mediate electron transfer to the P450 heme (Munro *et al.*, 2007). Less well studied is a small group of P450s that catalyze oxidative phenol coupling reactions on substrates containing phenolic groups (Isin & Guengerich, 2007). Molecular oxygen is again required, but no oxygen atom is incorporated into the product of the enzymic reaction, although there is again a requirement for electrons, which must be shuttled from NAD(P)H to the heme during the catalytic cycle.

Three bacterial class I cytochrome P450 enzymes called OxyA, OxyB and OxyC catalyze three key cross-linking reactions in the biosynthesis of glycopeptide antibiotics of the vancomycin/balhimycin family (Fig. 1). X-ray crystal

structures of OxyB and OxyC from the vancomycin producer *Amycolatopsis orientalis* confirmed that each contains a typical P450 fold, with a conserved cysteine residue acting as a proximal axial ligand for the heme (Zerbe *et al.*, 2002; Pylypenko *et al.*, 2003). The order of the cross-linking reactions in balhimycin biosynthesis has been defined through gene inactivation experiments in *Amycolatopsis balhimycina* (Süssmuth *et al.*, 1999; Bischoff *et al.*, 2001a, b; 2005). The first cross-link, introduced by OxyB, is an aryl-ether bridge (C–O–D ring) between the side chains of residues-4 and -6. The second cross-link (D–O–E ring) is introduced by OxyA, and the final biaryl link (the AB ring) is created by OxyC. *In vitro* experiments have shown that linear hexa- or heptapeptides attached as C-terminal thioesters to the pantetheinyl group of a peptide carrier protein (PCP) domain from the glycopeptide nonribosomal peptide synthetase (NRPS) are the preferred substrates for OxyB (Zerbe *et al.*, 2004; Woithe *et al.*, 2007). The first cross-link, therefore, is introduced while the peptide chain of the antibiotic is still attached to the NRPS assembly line. So far, *in vitro* assays with the two remaining cross-linking enzymes

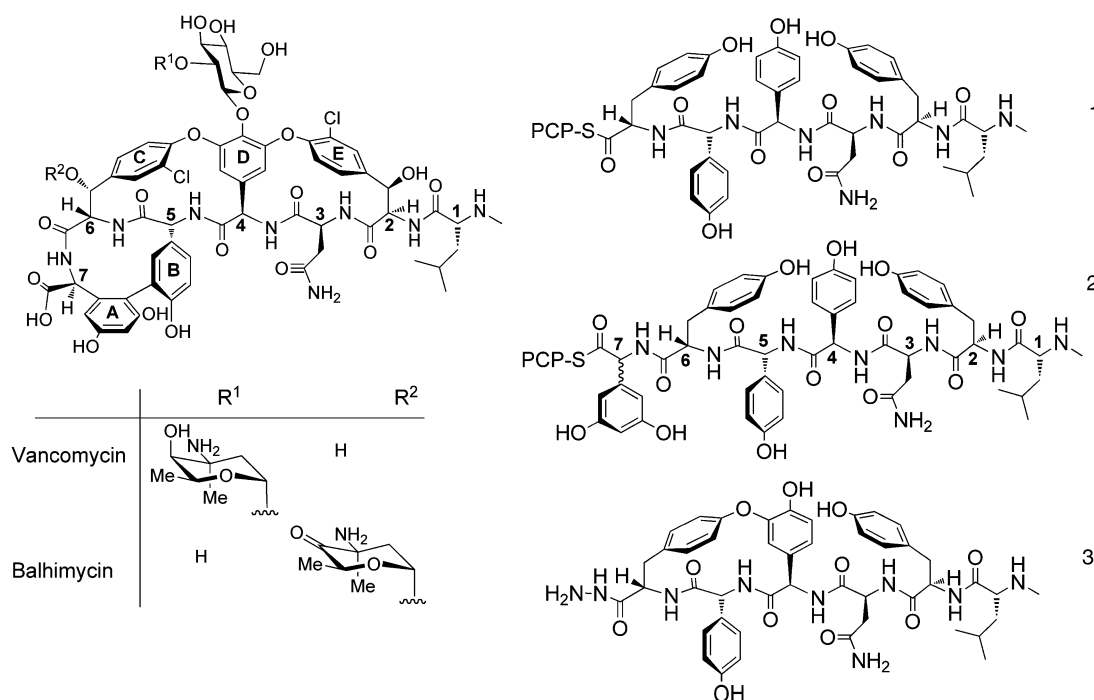


Fig. 1. Structures of vancomycin and balhimycin, the substrates (**1** and **2**) used for assays of OxyB, and the monocyclic hexapeptide product (**3**).

OxyA and OxyC have not been reported, and so the timing of these cross-linking steps remains undefined. One complicating factor for *in vitro* assays is that the natural electron transfer proteins for all three P450s have not been defined to date. The glycopeptide antibiotic gene clusters reported for balhimycin, chloroeremomycin, A40926, A47934 and teicoplanin do not contain Fd or FdR genes (Donadio *et al.*, 2005). Only the biosynthetic gene cluster for the related natural product complestatin contains a single Fd gene (*comK*) (Chiu *et al.*, 2001).

To advance *in vitro* studies of the cross-linking steps in glycopeptide antibiotic biosynthesis, we present efforts to identify and characterize Fd genes in the balhimycin producer *A. balhimycina*. The sequencing and annotation of the entire genome is currently underway. We describe *in silico* analyses, which reveal 11 different Fd genes in *A. balhimycina*. Furthermore, we demonstrate the production and purification of two of the newly identified Fds, as well as their ability to participate in electron transfers to OxyB from both *A. balhimycina* and *A. orientalis*.

Materials and methods

Bioinformatic analyses

The *A. balhimycina* DSM5908 genome was analyzed using a BLAST search carried out with six Fd sequences (NP-631171,

NP-629284, NP-631715, NP-625075, NP-628054 and NP-625924) from *Streptomyces coelicolor* A3(2) (Lamb *et al.*, 2002; Chun *et al.*, 2007), putidaredoxin (P00259) from *Pseudomonas putida* (Peterson *et al.*, 1990; Pochapsky *et al.*, 1994; Sevrioukova *et al.*, 2003) and the putative ferredoxin comFd (AAK81833) from the complestatin producer *Streptomyces lavendulae* (Chiu *et al.*, 2001). Eleven putative Fds, named balFd-I to balFd-XI, containing expect (E)-values $< 10^{-6}$, were identified in the whole genome of *A. balhimycina* (Table 1). Assignment of the iron-sulfur cluster type was achieved through a BLAST search of the nonredundant database and a comparison with known Fds. The sequences of the ferredoxins balFd-I to balFd-XI have been deposited in the EMBL database under the accession numbers FN594523–FN594533.

Cloning, expression and purification of recombinant *A. balhimycina* ferredoxins

The genomic DNA of *A. balhimycina* DSM5908 was used as a PCR template to amplify the genes coding for the putative [3Fe-4S] ferredoxins balFd-V and balFd-VII. The primers used are shown below (restriction sites underlined):
 5'-balFd-V: 5'-TAGACCATATGAAGGTTGTTGTCGACG-3'
 3'-balFd-V: 5'-ATCTACTCGAGGGCCTCTTCGAGGGC-3'
 5'-balFd-VII: 5'-TAGACCATATGAAGGTCACCGTGGACG-3'
 3'-balFd-VII: 5'-ATCTACTCGAGCGCGTCTCTCGCTCACC-3'

Table 1. Putative ferredoxins identified in the genome of *Amycolatopsis balhimycina*, showing the assigned name and accession number in the EMBL database, predicted number of amino acids and type of Fe-S cluster, as well as known ferredoxins showing high sequence similarities to each

Name	Amino acids (Fd cluster type)*	Significant matches in databases†			
		Species	Name	Protein identifier	% Identity
balFd-I	106 (7Fe)	<i>Mycobacterium smegmatis</i>	FdxA	gi 151568127	84
FN594530		<i>Streptomyces griseus</i>	7Fe	P13279	77
balFd-II	137 (7Fe)	<i>Mycobacterium smegmatis</i> MC2 155	FdxA	ABK72965	70
FN594531		<i>Streptomyces coelicolor</i>	Fdx2	NP_629284	56
balFd-III	64 (3Fe)	<i>Saccharopolyspora erythraea</i>	FdxB	CAM02117	48
FN594532		<i>Streptomyces avermitilis</i>	FdxB	BAC69321	45
balFd-IV	62 (3Fe)	<i>Streptomyces noursei</i>	NysM	AAF71770	59
FN594533		<i>Streptomyces nodosus</i>	AmphM	AAK73508	55
balFd-V	65 (3Fe)	<i>Streptomyces hygroscopicus</i>	HbmFdx	AAY28236	68
FN594523		<i>Streptomyces griseolus</i>	Fd-2	P18325	61
balFd-VI	76 (3Fe)	<i>Nocardia farcinica</i>	Fer	BAD55984	38
FN594529		<i>Streptomyces nodosus</i>	AmphM	AAK73508	37
balFd-VII	66 (3Fe)	<i>Streptomyces griseolus</i>	Fd-2	P18325	62
FN594528		<i>Streptomyces noursei</i>	NysM	AAF71770	55
balFd-VIII	64 (3Fe)	<i>Streptomyces griseolus</i>	Fd-2	P18325	53
FN594524		<i>Streptomyces hygroscopicus</i>	HbmFdx	AAY28236	50
balFd-IX	63 (3Fe)	<i>Mycobacterium tuberculosis</i>	FdxD	CAA17740	52
FN594525		<i>Nocardia farcinica</i>	Fer	BAD55378	52
balFd-XI	71 (3Fe)	<i>Streptomyces peucetius</i>	Fdx876	ACE73825	44
FN594527		<i>Streptomyces avermitilis</i>	FdxF	NP_827030	40
balFd-X	103 (2Fe)	<i>Rhodococcus</i> sp. RHA1	Fer2	YP_708187	70
FN594526		<i>Nocardia farcinica</i>	Fer2	YP_119598	62

*The 7Fe ferredoxins should contain a [4Fe-4S] and a [3Fe-4S] cluster.

†Identified by searching the nonredundant protein sequences (nr) database using each balFd sequence.

After digestion with NdeI and XhoI, the fragments were cloned between the NdeI/XhoI sites of plasmid pET22b (Novagen) for expression as C-terminal His₆-tagged fusion proteins. The nucleotide sequence of each insert was confirmed by sequencing.

For production in *Escherichia coli* Rosetta2(DE3)pLysS (balFd-V) or Origami2(DE3) (balFd-VII), terrific broth medium (400 mL) supplemented with antibiotics and FeSO₄ (0.5 mM) was induced with isopropyl-β-D-thiogalactopyranoside (0.2 mM), and shaking at 30 °C for 6 h. Cell pellets in buffer-A (25 mL, 50 mM sodium phosphate pH 7.4, 300 mM KCl, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 0.05% 2-mercaptoethanol and 5% glycerol) were disrupted by sonication and the cell debris was removed by centrifugation. The supernatant was loaded onto a nickel (Ni)-nitrilotriacetic acid (NTA) column (10 mL) in buffer-B (50 mM sodium phosphate, pH 7.4, 300 mM KCl and 20 mM imidazole). After washing, C-terminal His₆-tagged proteins were eluted with buffer-C (50 mM sodium phosphate, pH 7.4, 300 mM KCl and 300 mM imidazole). The brown ferredoxins were dialyzed against Tris/HCl buffer (50 mM, pH 7.5, 1 mM EDTA and 20% glycerol) and concentrated by ultrafiltration (3 kDa cut-off, Millipore). Size exclusion chromatography (Superdex 75 10/300 GL, GE Healthcare) was carried out,

eluting with Tris/HCl buffer (50 mM, pH 7.5, 1 mM EDTA and 20% glycerol). The purified proteins showed a single band by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and yielded a MALDI-MS corresponding to the His₆-tagged proteins with loss of the [3Fe-4S] cluster (balFd-V: $m/z = 7826$ [M+H]⁺, calc. 7826.6; balFd-VII: $m/z = 7897$ [M+H]⁺, calc. 7896.6). The amounts of iron- and acid-labile sulfide per balFd-V and balFd-VII were determined following published procedures (Beinert, 1983; Fish, 1988). The iron content was also determined by atomic adsorption spectroscopy (AAS).

Spinach Fd (spinFd), *E. coli* FdR (ecoFdR) and flavodoxin (ecoFld) were produced, following the methods described earlier (Woithe *et al.*, 2007).

Production of P450s

The production and characterization of P450s followed the methods described earlier (Zerbe *et al.*, 2002; Woithe *et al.*, 2007). Each purified protein showed a single band of c. 45 kDa by SDS-PAGE, and yielded an electrospray MS spectrum consistent with the expected protein sequence minus the N-terminal methionine residue (data not shown). Furthermore, the UV-Vis spectrum of each P450 showed a

Soret peak at 420 ± 1 nm and α/β bands around 569/537 nm.

Carbon monoxide (CO)-binding assays

Assays contained P450 (10 μ M), NADPH (0.5 mM), glucose-6-phosphate (0.5 mM), glucose-6-phosphate dehydrogenase (0.5 U) in Tris-HCl buffer (50 mM, pH 7.5), with ecoFdR (20 μ M) and one of: (A) spinFd (10 μ M); (B) ecoFdI (10 μ M); (C) balFd-V (10 μ M); (D) balFd-VII (10 μ M). The solution was divided between two cuvettes and CO was bubbled through the sample cuvette for 20 s. Difference spectra were recorded from 600 to 350 nm over 120 min.

Activity assays

Production and purification of *apo*-PCP, and the synthesis of peptide-PCP conjugates (1 and 2, Fig. 1), were as described previously (Woithe *et al.*, 2007). The assay, containing P450 (5–10 μ M), a reduction system [Fd or ecoFdI (10 μ M), ecoFdR (20 μ M)], NADPH (1 mM), glucose-6-phosphate (1 mM), glucose-6-phosphate dehydrogenase (0.5 U) and a PCP-bound substrate (50–100 μ M) in Tris/HCl buffer (1 mL, 50 mM, pH 7.5), was incubated at 30 °C for 60 min. Protein was precipitated with 1/10 volume of trichloroacetic acid (TCA) (6 M), and the resulting pellet was resuspended in 400 μ L Tris/HCl buffer (50 mM, pH 7.5) containing 2.5% v/v hydrazine. After incubation at 30 °C for 30 min, the proteins were precipitated with TCA and separated from the supernatant containing the peptide hydrazides, which were analyzed by analytical reverse-phase HPLC [Agilent Zorbax Eclipse XDB (RP C18, 250×4.6 mm, 5 μ M particle, 80 Å)] using a gradient of 5–40% MeCN/0.1% TFA in water/0.1% TFA over eight column volumes at a flow rate of 1 mL min⁻¹.

Results and discussion

In silico analysis of the *A. balhimycina* genome for ferredoxins

Fds are typically small acidic proteins containing one or two [Fe-S] clusters. The three most common clusters found in one-electron transfer reactions are [2Fe-2S], [3Fe-4S] and [4Fe-4S]. A family of 7Fe Fds found in bacteria contains both [3Fe-4S] and [4Fe-4S] clusters within the same polypeptide chain (Meyer, 2008). The draft genome of *A. balhimycina* DSM5908 was analyzed *in silico* for putative Fds. Table 1 summarizes the 11 newly identified putative Fds from *A. balhimycina*, which were named balFd-I to balFd-XI (Palmer & Reedijk, 1991).

Sequence analyses of balFd-I and balFd-II indicate high sequence homologies (56–84% identity) toward the family of 7Fe Fds (Fig. 2a), including FdxA from *Mycobacterium*

smegmatis, whose crystal structure reveals one [3Fe-4S] and one [4Fe-4S] cluster (Ricagno *et al.*, 2007). The seven Cys residues that bind the [3Fe-4S][4Fe-4S] clusters in FdxA from *M. smegmatis* are strictly conserved in balFd-I and balFd-II. *In vitro* activity for the 7Fe Fd from *Streptomyces griseus* has been demonstrated with P450soy (Trower *et al.*, 1990).

Eight putative Fds (balFd-III to balFd-IX; balFd-XI) show moderate to high sequence identities toward a variety of [3Fe-4S] Fds. In these cases, three strictly conserved Cys residues are involved in binding the [3Fe-4S] cluster (shown for balFd-V and balFd-VII in Fig. 2b), which strengthens the assignment of these balFds as [3Fe-4S] Fds. Interestingly, balFd-IV, balFd-V, balFd-VII and balFd-VIII show high sequence homologies toward Fds that can either be found adjacent to a P450 monooxygenase in a biosynthetic gene cluster [NysM (Brautaset *et al.*, 2000), AmphM (Caffrey *et al.*, 2001) and HbmFdx (Rascher *et al.*, 2005)], or that has been shown to copurify with a P450 enzyme [Fd-2 from *Streptomyces griseolus* (O'Keefe *et al.*, 1991)]. These results suggest that the newly identified Fds might play a role in supporting the activity of P450 enzymes in *A. balhimycina*.

The balFd-X exhibits high sequence similarities toward [2Fe-2S] proteins (Table 1 and Fig. 2c), in particular, Pdx from *P. putida*, the natural redox partner of P450cam, with which it shares a sequence identity of 42% and contains the same four highly conserved cysteine residues involved in binding the Fe-S cluster (Matsubara & Saeki, 1992). The genomes of *S. coelicolor* A3(2) (Bentley *et al.*, 2002), *Streptomyces avermitilis* (Ikeda *et al.*, 2003), *S. griseus* (Ohnishi *et al.*, 2008), *Mycobacterium tuberculosis* (Cole *et al.*, 1998) and *Saccharopolyspora erythraea* (Oliynyk *et al.*, 2007) do not contain proteins with similarities to Pdx or balFd-X.

Shotgun sequencing of the genome of *A. balhimycina* is finished, but manual annotation is still ongoing. Most importantly, no gene encoding a putative Fd can be found approximately 360 kbp upstream or downstream of the balhimycin biosynthetic gene cluster. However, balFd-V and balFd-VII are each located in close vicinity to other putative P450s, as well as FdRs, of unknown function.

Production and characterization of recombinant ferredoxins

The putative 7Fe Fd balFd-I, three of the putative [3Fe-4S] Fds balFd-IV, balFd-V and balFd-VII, as well as the presumed [2Fe-2S]-containing balFd-X were selected here for more detailed biochemical studies. Attempts were made to produce each of the five putative Fds in *E. coli* as a C-terminal His₆-tagged recombinant protein. However, only balFd-V and balFd-VII could be produced efficiently as cofactor-containing proteins. The production of balFd-I

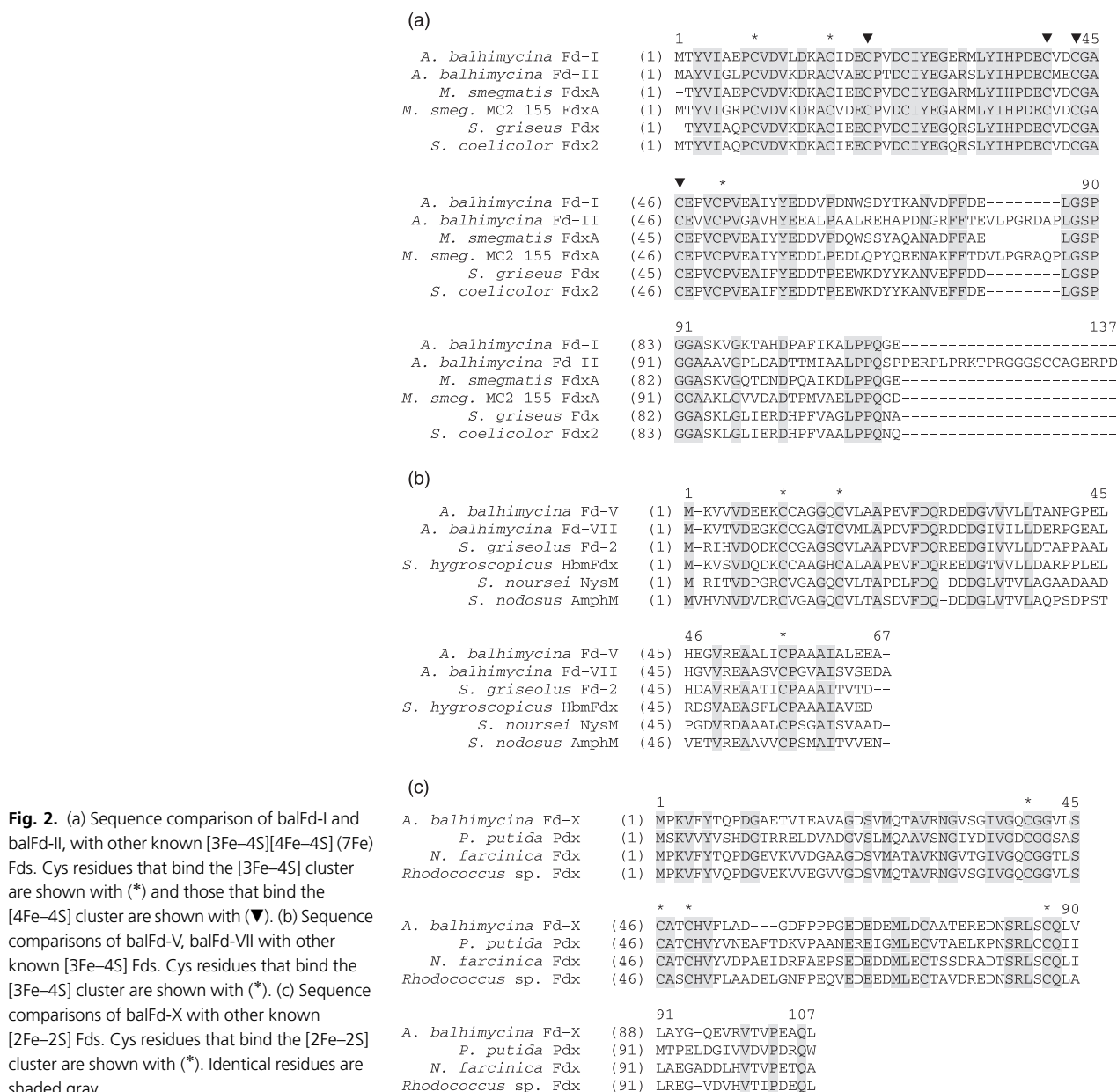


Fig. 2. (a) Sequence comparison of balFd-I and balFd-II, with other known [3Fe-4S][4Fe-4S] (7Fe) Fds. Cys residues that bind the [3Fe-4S] cluster are shown with (*) and those that bind the [4Fe-4S] cluster are shown with (▼). (b) Sequence comparisons of balFd-V, balFd-VII with other known [3Fe-4S] Fds. Cys residues that bind the [3Fe-4S] cluster are shown with (*). (c) Sequence comparisons of balFd-X with other known [2Fe-2S] Fds. Cys residues that bind the [2Fe-2S] cluster are shown with (*). Identical residues are shaded gray.

and balFd-IV in *E. coli* Rosetta2(DE3)pLysS yielded colorless His₆-tagged recombinant proteins, lacking the chromophore expected from intact Fe-S clusters. Overexpression of the balFd-X gene in *E. coli* yielded no recombinant protein with the expected mass. Further studies on these Fds were not pursued here.

The production of balFd-V and balFd-VII yielded red-brown-colored *holo*-proteins that were purified by Ni-NTA and gel filtration chromatography. Each protein eluted from a gel filtration column (Superdex 75 10/300 GL, GE Healthcare) with an apparent mass of about 24–26 kDa (O'Keefe *et al.*, 1991). Both proteins were ≥90% homogeneous

by SDS-PAGE and analytical reverse-phase HPLC, and yielded ions consistent with the expected masses for the *apo*-forms by MALDI-MS (for balFd-V $m/z = 7826$ [M+H]⁺, calc. 7826.6; for balFd-VII $m/z = 7897$ [M+H]⁺, calc. 7896.6).

The recombinant balFd-V and balFd-VII showed broad UV-Vis absorption maxima at 280–300 nm and 412 nm (Fig. 3), which are typical for oxidized [3Fe-4S] or [4Fe-4S] Fds (Jouanneau *et al.*, 1990; O'Keefe *et al.*, 1991). By comparison, the recombinant His₆-tagged [2Fe-2S] spinFd showed the expected absorbance maxima at 275, 328, 420 and 463 nm (Armengaud *et al.*, 2000).

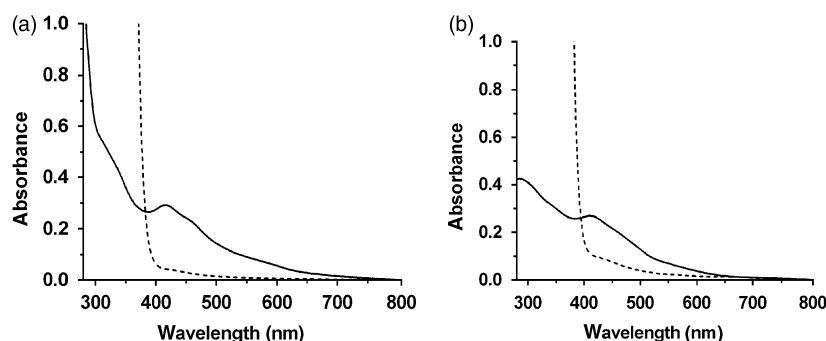


Fig. 3. UV-Vis absorption spectra of balFd-V (a) and balFd-VII (b) in Tris/HCl buffer (50 mM, pH 7.5) (solid lines), and after reduction with sodium dithionite (broken lines).

Extinction coefficients for balFd-V and balFd-VII were determined using AAS to establish the iron content, with the assumption that one [3Fe-4S] cluster is present in each polypeptide chain ($\epsilon_{412} = 18\,300\text{ M}^{-1}\text{ cm}^{-1}$ for balFd-V and $14\,660\text{ M}^{-1}\text{ cm}^{-1}$ for balFd-VII). The values found are close to those reported for two Fds from *S. griseolus*: Fd-1 ($\epsilon_{410} = 17\,000\text{ M}^{-1}\text{ cm}^{-1}$) and Fd-2 ($\epsilon_{410} = 20\,100\text{ M}^{-1}\text{ cm}^{-1}$) (O'Keefe *et al.*, 1991). The quantification of acid-labile sulfide was also carried out using a colorimetric assay (Beinert, 1983). Under optimized conditions, the assays yielded 4.01 ± 0.5 and $3.84 \pm 0.5\text{ mol S mol}^{-1}$ protein for balFd-V and balFd-VII, respectively.

Ferredoxin-mediated reduction of P450 heme iron

The OxyB P450 enzymes from the vancomycin biosynthetic gene cluster of *A. orientalis* (vanOxyB) and from the balhimycin biosynthetic gene cluster of *A. balhimycina* (balOxyB) were used for CO-binding and activity assays. Upon reduction of the P450 heme iron from Fe(III) to Fe(II) using sodium dithionite and exposure to CO, both enzymes showed a characteristic Soret absorbance maximum at 450 nm. Following formation of the Fe(II)-CO complex, a new species is formed over 1–2 h, absorbing at about 420 nm, the so-called P420 form, which arises by protonation of the proximal cysteine thiolate ligand of the heme iron (Perera *et al.*, 2003; Dunford *et al.*, 2007).

CO-binding assays were also performed with each P450 and the primary electron transfer proteins ecoFdr and one of spinFd, balFd-V, balFd-VII or ecoFld. These assays monitor the ability of each donor to transfer one electron to the P450 heme Fe(III), followed by binding of CO to the Fe(II) form. Although the proteins tested have diverse species origins (spinach, *E. coli* and *A. balhimycina*) and cofactor types ([2Fe-2S], FMN, [3Fe-4S]), all four were able to reduce the Fe(III) heme of vanOxyB, albeit to different extents. The plant spinFd rapidly (< 6 min) led to up to 75% conversion (relative to dithionite) to the vanOxyB P450-form, whereas with ecoFld, a maximum of 40% conversion was attained only after 40 min. A rapid

formation of the P450 from vanOxyB (< 10 min) was also observed using either Fd from *A. balhimycina*, with 60% conversion with balFd-V and 45% with balFd-VII.

The OxyB enzymes from the balhimycin and vancomycin pathways share 88% sequence identity. Nevertheless, some differences were observed between these enzymes in their abilities to accept electrons from the four primary redox partners. Thus, both balOxyB and vanOxyB are rapidly (< 5–6 min) and efficiently (80%) converted into the P450 form by spinFd, but the emergence of the P450 form with balFd-V and balFd-VII was slower and reached at best 40% (balFd-V) and 20% (balFd-VII) of the response seen with sodium dithionite. With balOxyB, essentially no reduction was observed using ecoFld.

These CO-binding assays do not indicate whether or not a second electron transfer can occur to the heme as required in the full P450 catalytic cycle. For example, spinFd can donate the first electron to camphor-bound P450cam, but allows no hydroxylation of substrate (Lipscomb *et al.*, 1976). To address this point, assays were also carried out with peptide substrates of OxyB.

Ferredoxin-mediated catalytic activity

The assay for the catalytic activity of OxyB is that described in an earlier work (Zerbe *et al.*, 2004; Woihte *et al.*, 2007, 2008; Geib *et al.*, 2008). The substrates used are the model hexa- and heptapeptides 1 and 2 (Fig. 1), which are closely related to the expected intermediates occurring during glycopeptide biosynthesis. Each peptide is covalently linked as a C-terminal thioester to an isolated recombinant PCP domain from the seventh module of the vancomycin NRPS. For ease of synthesis (Li & Robinson, 2005), these model peptides contain tyrosine at positions -2 and -6, rather than β -hydroxy-*meta*-chlorotyrosine (see Fig. 1). Standard conditions were used for all assays, so that a comparison of turnover efficiencies could be obtained from the extent of linear peptide conversion into the corresponding monocyclic product. The relative amounts of remaining substrate and monocyclic (C-O-D ring) product formed were determined after removal of the peptide from the PCP with

hydrazine, and HPLC and ESI-MS analysis of the corresponding peptide hydrazides (e.g. the hexapeptide hydrazide **3**, Fig. 1).

The results obtained in assays with vanOxyB and balOxyB are summarized in Table 2. Intriguingly, all of the electron transfer proteins are able to effectively donate two electrons to vanOxyB during the catalytic cycle, using the hexapeptide-PCP (**1**) as a substrate, with conversions to monocyclic product (**3**), under the standard conditions, ranging from 60 to over 90%. The heptapeptide-PCP (**2**, Fig. 1), however, is less efficiently converted into the corresponding monocyclic product, with conversions from 10% to 60% observed. It is important, however, to note that this heptapeptide substrate (**2**) is a mixture of inseparable diastereomers (which arise during the synthesis of the substrate) differing in configuration at C(α) in residue-7. The results also suggest a more favorable interaction between vanOxyB and spinFd or balFd-VII, than between vanOxyB and ecoFld or balFd-V.

Similar findings were obtained in activity assays using balOxyB. In this case, however, the differences in substrate turnover achieved with the four electron transfer proteins, and between the hexa- and heptapeptide substrates, are more pronounced. Assays with balOxyB and ecoFld or balFd-V showed only a marginal turnover of hexapeptide (**1**) to a monocyclic product (**3**). However, with spinFd and especially with balFd-VII, significant cyclization of the substrate was observed (Table 2), with conversion of hexapeptide to monocycle similar to that seen in assays with vanOxyB. However, the turnover of heptapeptide (**2**) was significantly lower, with the best result being 15% conversion to a monocyclic product achieved with balFd-VII. These results suggest a higher discrimination between the hexa- and heptapeptides, with the hexapeptide being more strongly favored as a substrate by balOxyB. Finally, these findings also indicate degeneracy in the ability of various

different Fds to support the catalytic activities of P450 coupling enzymes from different glycopeptide-producing organisms. This property may well make it difficult to assign a specific function to each of the individual Fds identified in the *A. balhimycina* genome, at least using *in vitro* assays. On the other hand, this flexibility should be an advantage in facilitating more detailed *in vitro* studies of these interesting cytochrome P450 cross-linking enzymes.

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Table 2. Activity assays with vanOxyB and balOxyB using various electron transfer proteins

P450	Reduction system	Monocyclic product*	
		Substrate: 1 (%)	Substrate: 2 (%)
vanOxyB	spinFd/ecoFldR	> 90	~60
	ecoFld/ecoFldR	~70	~15
	balFd-V/ecoFldR	~60	~10
	balFd-VII/ecoFldR	~80	~40
balOxyB	spinFd/ecoFldR	~45	~5
	ecoFld/ecoFldR	< 5	< 1
	balFd-V/ecoFldR	< 5	< 1
	balFd-VII/ecoFldR	~75	~15

The conversions of substrates **1** and **2** (Fig. 1) into the corresponding monocyclic products are shown.

*Amount of monocyclic hydrazide product formed after 1 h as determined by HPLC.

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